ORIGINAL ARTICLE

De novo 19q13.42 duplications involving *NLRP* gene cluster in a patient with systemic-onset juvenile idiopathic arthritis

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Systemic-onset juvenile idiopathic arthritis (s-JIA) is a rare inflammatory disease classified as a subtype of chronic childhood arthritis, manifested by spiking fever, erythematous skin rash, pericarditis and hepatosplenomegaly. The genetic background underlying s-JIA remains poorly understood. To detect disease-related copy number variations (CNVs), we performed single-nucleotide polymorphism array analysis in 50 patients with s-JIA. We detected many CNVs, but most of them were inherited from either of normal-phenotype parents. However, in one patient, we could identify two *de novo* microduplications at 19q13.42 with the size of 77 and 622 kb, separated by a 109-kb segment of normal copy number. The duplications encompass *NLRP* family (*NLRP2*, *NLRP9* and *NLRP11*) as well as *IL11* and *HSPBP1*, all of which have an important role in inflammatory pathways. These genes may significantly contribute to the pathogenesis of s-JIA.

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INTRODUCTION

Juvenile idiopathic arthritis (JIA) is a chronic rheumatic disease of childhood. Approximately 11% of JIA patients show systemic-onset JIA (s-JIA),¹ which is clinically manifested by spiking fever, erythematous skin rash, pericarditis and hepatosplenomegaly in addition to arthritis. Abnormal innate immunity involving cytokines such as interleukin (IL)-1 and IL-6, neutrophils and monocytes/macrophages may play a major role in the pathogenesis of s-JIA. One of the major features of s-JIA is its progression to macrophage activation syndrome. On the basis of all these evidences, it is now generally accepted that s-JIA should be classified as an autoinflammatory syndrome rather than a classical autoimmune disease.

Two genetic factors, *HLA* and *PTPN22*, have been found as JIA susceptibility genes in multiple populations.² For example, *HLA-B27*, *HLA-DR1* and *HLA-DR4* have been reported to increase risk for polyarticular JIA.³ However, such associations are seen mostly in polyarticular JIA, but not in s-JIA. Other genes including *MIF*, *IL6*, *IL10* and *TNF* are reported to be associated with s-JIA in different populations and subtypes,^{4–8} although these genes account for only a small part of the total genetic contribution to JIA. Therefore, the genetic background underlying the s-JIA remains mostly undetermined.

We performed genome-wide copy number variations (CNVs) analysis in s-JIA patients. Two *de novo* microduplications at 19q13.42 encompassing 77 and 622 kb were identified in one patient by single-nucleotide polymorphism (SNP) array 6.0 and confirmed by other methods. The duplications encompass *NLRP* (Nucleotide-binding oligomerization domain, Leucine rich Repeat and Pyrin domain) family (*NLRP2*, *NLRP9* and *NLRP11*), which have important roles in inflammatory processes as well as *IL11*, which was reported to correlate with arthritis severity in s-JIA patients. This is the first report of *de novo* CNVs in relation to s-JIA.

MATERIALS AND METHODS

Subjects

A total of 50 patients with s-JIA, which was refractory to conventional treatment and was treated with tocilizumab, were enrolled with informed consent based on the IRB-approved protocols at Yokohama City University Hospital. There were no family histories in each patient. Genomic DNA of peripheral blood leukocytes from all patients were isolated using DNA isolation systems (Quick Gene-800; Fujifilm, Tokyo, Japan).

Whole-genome SNP array and custom array analyses

To detect CNVs, two different SNP array platforms, the Genechip Human Mapping 250K array (Affymetrix, Santa Clara, CA, USA) (for 23 patients) and

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the Genome-wide Human SNP array 6.0 (Affymetrix) (for the other 27 patients), were used following the manufacturer's protocols. In brief, for the Genome-wide Human SNP array 6.0, 500-ng DNA was digested with *Nsp1* and *Sty1* (only *Nsp1* was used for 250K array). The adaptors were ligated to the digested DNA, and the ligation-mediated polymerase chain reaction (PCR) with single primer was performed. PCR products were purified by magnetic beads (Ampure; Beckman Coulter Company, Beverly, MA, USA). Microcon-YM100 (Millipore Corporation, Bedford, MA, USA) was used for purification for the 250K array. The product was fragmented, end-labeled and hybridized to an array. CNAG3.0 (ref. 9), Genotyping Console (Copy Number Analyzer for GeneChip) (Affymetrix) and Partek Genomic Suite (Partek, St Louis, MO, USA) were used to validate copy number alterations. The qualities of the results were high in every sample (250K array: SNP call rate >95%, multifactor dimensionality reduction >99%, (multifactor dimensionality reduction—minimum candidate region) <5%; SNP array 6.0: contrast quality control >2, quality control call rate >93%, median absolute pairwise difference (MAPD) <0.4).

We also performed custom high-density oligonucleotide array-comparative genomic hybridization (CGH) analysis using custom-made 4×72K array (Roche NimbleGen, Madison, WI, USA) based on the manufacturer's protocol. This slide covered the genomic region of chromosome 19 between 59 510 019 and 61 490 039 bp with 71 891 probes. The average probes spacing are 20 bp, and the probes encompassing two duplication regions (60 144 400–60 429 000 and 60 860 000–61 051 000 bp) are 10 bp. DNA (500 ng) was sonicated with the condition of 10 s, power level 1, pulse 1 s and duty 50% using SONIFIRE-250D (Branson, Danbury, CT, USA). Patient's DNA was labeled with Cy3-random nonamers and patient's parent (father or mother) was labeled Cy5-random nonamers. Dyes were swapped in the combination of father/patient and mother/patient (patient's DNA with Cy5 and parent DNA with Cy3). Labeled DNA was hybridized at 42 °C for 16–20 h, and washed. The data analysis was carried out by NimbleScan (Roche NimbleGen) and visualized by SignalMap (Roche NimbleGen).

Fluorescence *in situ* hybridization analysis

Fluorescence *in situ* hybridization (FISH) analysis was performed on metaphase chromosomes and interphase nuclei of the patient's and parental peripheral blood leukocytes. The bacterial artificial chromosome clone, RP11-384F2, mapped to the duplicated segment was used as a probe (60 374 564–60 616 811 bp at 19q13.42, UCSC Genome Browser on Human, March 2006; hg18). Bacterial artificial chromosomes were labeled with Cy3-dUTP (Invitrogen, Carlsbad, CA, USA) by Nick translation kit (Vysis, Des Plaines, IL, USA). Probe-hybridization mixtures (16 µl) were mounted on chromosomes/nuclei, incubated at 37 °C for 16–72 h and washed. Chromosomes and nuclei were counterstained with 4',6-diamidino-2-phenylindol containing antifade solution. Fluorescence photomicroscopy was performed under an AxioCam MR CCD fitted to Axioplan2 fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

Quantitative real-time PCR to confirm copy number changes

The deletion breakpoints were analyzed using genomic DNAs by quantitative real-time qPCR with Quantifast SYBR Green PCR kit on Rotor-Gene™ 6200 HRM (Corbett Life Science, Sydney, Australia). The delta-delta Ct relative quantitative method was employed according to the manufacturer's protocol. Averages of duplicates were calculated by ROTOR-GENE 6000 SERIES software (Corbett Life Science).

Real-time reverse transcription-PCR

Lymphoblastoid cell lines established from the patient and her parents, and three normal controls were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1×antibiotic–antimycotic (Invitrogen) and 8 µg ml⁻¹ tylosin (Sigma, St Louis, MO, USA) at 37 °C in a 5% CO₂ incubator. Total RNA was independently extracted twice using RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA). Total RNA (2 µg) was subjected to reverse transcription (RT) using PrimeScript first-strand synthesis kit with random hexamers (Takara, Ohtsu, Japan). Quantitative real-time RT-PCR was performed using TaqMan Gene expression assays (Applied Biosystems) (FAM label: Hs00174148_m1 for *IL11*, Hs00215284_m1 for *NLRP2*, Hs00603423_m1 for

NLRP9, Hs00935472_m1 for *NLRP11*; VIC-label: Human ACTB (β-actin) Endogenous Control). Multiplex quantitative real-time RT-PCR was carried out by the two standard curve methods on RoterGene-6200 HRM (Corbett Life Science). Relative gene expression was calculated in comparison with that of the reference (used in standard curve) cDNA. The data from duplicated experiments using two distinctive RNA samples were averaged and the standard deviation was calculated.

RESULTS

Clinical features of the proband with *de novo* duplications

The proband is a 15-year-old female subject who developed s-JIA at 2 years old, with swelling of her left knee and limping. She was resistant to antibiotics, anti-inflammatory therapy, methylprednisolone pulse therapy, cyclosporin A, mizoribine, methotrexate and azathioprine. As she was resistant to all the conventional therapies, she was admitted to Yokohama City University Hospital at 9 years old and received tocilizumab (anti-IL-6 receptor antibody) therapy. After starting tocilizumab, her condition got recovered and stable since then.

Whole-genome oligonucleotide SNP array

We performed analysis of 50 patients of s-JIA using whole-genome oligonucleotide SNP array (250K *Nsp1* or SNP 6.0; Affymetrix). The total copy number abnormalities were 9 deletions and 12 duplications (Table 1).

Confirmation of CNVs by FISH and qPCR

Copy number changes were confirmed by other methods like FISH or qPCR and their origin was also checked using parental DNA if available. Most of the copy number abnormalities were of either paternal or maternal origin (Table 1), but two duplications at 19q13.42 on the patient (ID1395) occurred *de novo*, being confirmed by FISH analysis (Figure 1) and qPCR (data not shown).

Custom array-CGH

In addition, we performed custom oligonucleotide 72K array-CGH (Roche NimbleGen), to check the precise size of the duplications. CGH was performed using the following combination of test DNA/reference DNA: patient/father and patient/mother. Dye-swap analysis was also performed (Figure 2). As a result, two *de novo* microduplications at 19q13.42 with the size of 77 kb (Genome browser version hg18, chromosome 19 coordinates: 60 190 370–60 267 627 bp) and 622 kb (60 377 092–60 991 185 bp), separated by a 109-kb segment of normal copy number (60 267 627–60 377 092 bp), were identified. Two sets of test DNA/reference DNA combinations consistently showed the duplications, indicating that the duplications occurred *de novo*. Parentage of the family was confirmed using microsatellite markers (data not shown). Similar duplication was never deposited to the DECIPHER database (<http://decipher.sanger.ac.uk/syndrome>), although only one overlapping deletion was found in a patient with autism.

Expression analysis of duplicated genes

IL11, *NLRP2*, *NLRP9* and *NLRP11* were chosen to see their expression as they were mapped to the duplication. Low expression of *IL11* and *NLRP9* in lymphoblasts hindered the proper evaluation. *NLRP2* and *NLRP11* did show variable expression patterns depending on lymphoblastoid cells; thus, it was indeed difficult to see the effect of duplication of these genes (data not shown).

DISCUSSION

We identified two *de novo* microduplications in one out of 50 s-JIA patients. The duplications contain several interesting genes, including

Table 1 CNVs found in s-JIA patients

Patient ID	CNV overlap (%)	Size	Deletion	Duplication	Confirmation	Inheritance	Array type
1239	Partial CNV	267 kb		dup(3)(p12.2)	qPCR	Not available	250K
1247	0	275 b	del(11)(q21)		FISH	Inherit (father)	250K
1285	0	235 kb		dup(18)(q23)	qPCR	Inherit (father)	250K
1287	Partial CNV	1.58 mb	del(2)(q13)		FISH	Inherit (mother)	250K
	0	259 kb		dup(3)(p26.1)	qPCR	Inherit (mother)	
1317	0	13 kb	del(16)(q24.1)		qPCR	Not available	SNP 6.0
1333	0	93 kb		dup(5)(q34)	qPCR	CNV	SNP 6.0
1344	36	205 kb		dup(15)(q13q15.1)	qPCR	Inherit (father)	SNP 6.0
1350	0	50 kb	del(4)(q34.2)		qPCR	Not available	SNP 6.0
1361	0	128 kb	del(2)(q13)		qPCR, FISH	Inherit (mother)	SNP 6.0
1383	0	362 kb		dup(2)(q11.2)	qPCR, FISH	Inherit (mother)	SNP 6.0
	0	558 kb		dup(2)(q11.2)	qPCR, FISH	Inherit (mother)	
1395	27	622 kb		dup(19)(q13.42)	qPCR, FISH	<i>De novo</i>	SNP 6.0
	58	77 kb		dup(19)(q13.42)	qPCR, FISH	<i>De novo</i>	
1406	0	169 kb	del(1)(q25.3)		FISH	CNV	SNP 6.0
1407	0	144 kb		dup(13)(q12.11)	qPCR	Inherit (mother)	SNP 6.0
1433	0	13 kb	del(2)(q33.1)		qPCR	Inherit (father)	SNP 6.0
1434	42	5.5 mb	del(10)(q11.21q11.23)		FISH	Inherit (mother)	SNP 6.0
1439	62	906 kb		dup(1)(q43)	qPCR	Not available	SNP 6.0
1620	0	695 kb		dup(3)(q26.31)	qPCR	Not available	SNP 6.0
1669	0	85 kb	del(16)(q24.1)		qPCR	Not available	SNP 6.0

Abbreviations: CNV, copy number variation; FISH, fluorescence *in situ* hybridization; qPCR, quantitative real-time polymerase chain reaction; s-JIA, systemic-onset juvenile idiopathic arthritis; SNP, single-nucleotide polymorphism.

CNV, overlap shows overlapping ratio of reported CNVs. Inheritance indicates parental origin of CNVs. Not available: parental samples were unavailable.

De novo change is shown in bold.

Figure 1 Fluorescence *in situ* hybridization (FISH) analysis using a bacterial artificial chromosome (BAC) clone, RP11-384F2, on cells of the family. (a) Interphase nucleus of the patient, (b) metaphase chromosomes of the patient and (c, d) father's and mother's interphase nuclei, respectively. Arrowhead indicates double signals showing duplication at 19q13.42 and arrow indicates a single signal of normal chromosome.

The *NLRP* family is composed of 14 members, including *NLRP2*, *NLRP9* and *NLRP11* (ref. 10). Most *NLRPs* are encoded by two gene clusters on chromosome 11p15 (*NLRP6*, *NLRP10* and *NLRP14*) and 19q13.4 (*NLRP2*, *NLRP4*, *NLRP5*, *NLRP7*, *NLRP8*, *NLRP9*, *NLRP11*, *NLRP12* and *NLRP13*) (ref. 10). *NLRPs* are evolutionally conserved through *Caenorhabditis elegans*, *Drosophila melanogaster*, rat, mouse and human. *NLRP1* and *NLRP3* are known as components of the inflammasome implicated in early detection of extracellular pathogens and intracellular noxious compounds and driving inflammatory and immune responses.¹¹ Germline mutations in *NLRP3* and *NLRP12* are associated with hereditary periodic fever syndromes.^{12,13} Although the function of *NLRP9* and *NLRP11* is not well understood, *NLRP2* is suggested to function as a modulator of macrophage nuclear factor- κ B activation and caspase-1 activation,¹⁴ which may cause inflammation. All the evidences support that duplications of the *NLRP* gene cluster may significantly contribute to s-JIA pathogenesis in the patient.

IL-6 and IL-11 bind to their own ligand-specific receptor (IL-6R or IL-11R) and recruit a homodimer of gp130, which is responsible for intracellular signaling.¹⁵ The gp130 signaling cytokines contribute to inflammation and bone homeostasis. IL-6 and IL-11 play an important role in osteoclast development, such as promoting osteoclastogenesis and bone resorption in bone marrow cultures.^{16,17} IL-6 and IL-11 are capable of inducing osteoclast formation from peripheral blood mononuclear cells by a receptor activator of nuclear factor- κ B ligand-independent mechanism.¹⁸ IL-11 is produced by fibroblasts, mesenchyme-derived stromal cells of bone marrow, osteoblasts and chondrocytes.^{16,19} The expression levels of IL-11 are upregulated in patients with s-JIA compared with healthy children and positively correlated with the number of joints with active arthritis.²⁰ Duplication of IL-11 possibly associated with its higher expression in inflammatory cells is likely to be related to the arthritis in the patient, although the roles of IL-11 are not well clarified in rheumatoid arthritis pathogenesis and alteration of IL-11 expression could not be confirmed in the patient's lymphoblastoid cells owing to the low

expression level. It is possible that the anti-IL-6 receptor antibody therapy effectively suppressed the upregulated gp130 signaling in part by IL-11 duplication in this patient.

Heat-shock proteins (Hsps) are essential to prokaryotic and eukaryotic cellular organisms during intracellular (un)folding, assembly and translocation of proteins.²¹ Their synthesis is greatly enhanced in response to a variety of stressful stimuli such as temperature, hypoxia, infection and inflammation.²² Microbial Hsps are a potential inducer of crossreactive immune responses to host self-molecules that may lead to autoimmunity.²³ In several experimental models, T cells responding to Hsps play an important role in the regulation of peripheral tolerance and suppressing pathogenic immune response.²⁴ HspBP1 (Hsp70 binding protein 1) has an inhibitory effect in Hsp70-assisted refolding reactions in the cytosol,^{25,26} suggesting that HspBP1 plays an important role in regulating immune responses. HspBP1 duplication may alter immune responses in the s-JIA patient.

Genomic duplications can be pathogenic through both increased dosage (whole gene duplication, duplication of regulatory elements) and disruption of coding regions (intragenic duplications). Intronic duplications potentially disrupting the splicing machinery have also been reported to be pathogenic.²⁷ In our study, we found *de novo* duplications in one s-JIA patient, which involve many important genes for the regulation of the immune system. Other patients without any pathological CNVs in this study may have point mutations of a gene(s) mapped to the duplications, which may lead to upregulated gene expression causative for s-JIA as previously described in a different disease.²⁸ Further analysis is absolutely necessary.

In conclusion, this is the first report describing a possible relationship between CNVs and s-JIA, and we believe such abnormal genotypes are important to solve the pathogenesis of s-JIA.

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